REMARKS

Applicants respectfully request reconsideration and reexamination of the present application in light of the amendments and the remarks below. Claims 1-5, 7 and 8 are pending in this application.

Claims 1-5 and 7 have been amended, and new claim 8 has been added. No new matter has been added by the amendments or new claim. Support for the amendments and new claim is found throughout the application as originally-filed and from the pending and original claims. Exemplary support for the amendments and new claim is as follows. Support is provided with respect to a particular paragraph(s) and/or line numbers of the corresponding published U.S. application, Pub. No. 2002/0123845.

Support for "cell or tissue sample" of claim 1 can be found, for example, at lines 1-8 of paragraph 21.

Support for "selecting at least two markers of cancer that individually do not achieve sufficient specificity with regard to detecting cancer in said cells" of claim 1 can be found, for example, at lines 1-6 of paragraph 14. Support can also be found, for example, at lines 13-14 of paragraph 14.

Support for "a signaling reagent" of claim 1 can be found, for example, at paragraphs 27 through 30.

Support for "at least two molecular markers" of claim 1 can be found, for example, at paragraph 19.

Support for "within a constituent region of the tissue section" of claim 1 can be found, for example, at lines 9-11 of paragraph 16. Support can also be found, for example, at lines 13-15 of paragraph 19.

Support for "thereby identifying cancer cells and their precursors in the cell or tissue sample" of claim 1 can be found, for example, at lines 1-3 of paragraph 26.

Support for "further comprising the step of automatically processing the signal intensities into image information and consolidating said information into a proposed diagnosis using a linked diagnostic expert system" of claim 2 can be found, for example, at lines 1-7 of paragraph 24.

Support for "wherein the signaling reagents produce chromogenic color or fluorescence" of claim 3 can be found, for example, at line 3-4 of paragraph 18.

Support for new claim 8 can be found, for example, at lines 6-7 of paragraph 15.

These claim amendments are made to clarify the subject matter therein. Therefore, these amendments are submitted in order to place the claims in condition for allowance, and do not disclaim any subject matter to which the Applicants are entitled.

Rejection Under 35 U.S.C. § 102

The Examiner has rejected claims 1, 2, and 5 under 35 U.S.C. § 102(b) as being anticipated by Rao. et al., (Cancer Epidemiol. Biomarkers Prev.) (Paper No. 2-29-06, pages 2-3 and 6-7). Applicants respectfully traverse this rejection.

The claims are directed to automatable methods for identifying cancer cells and their precursors in a cell or tissue sample which comprises the steps of selecting at least two molecular markers that individually do not achieve sufficient specificity with regard to detecting cancer in said cells and contacting a cell or tissue sample with signaling reagents that specifically bind to the at least two molecular markers. The claimed methods further comprise the step of simultaneously detecting signal intensities from the markers within a constituent region of the tissue section. Further, the signal intensities of the detected signaling reagents are combined and accredited, thereby identifying cancer cells and their precursors in the cell or tissue sample.

The Examiner is respectfully pointed to M.P.E.P § 2131 which states that "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *See Verdegaal Bros. V. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987).

Rao, et al., relates to a study that evaluates the use of three biomarkers as potential indicators of breast cancer risk from tissue samples taken using fine-needle aspiration. The reference appears to report on, in particular, the evaluation of p53, G-actin and DNA as markers for assessing breast tissue biopsies. According to the reference, each of the markers are targeted using immunofluorescence labels and then are visualized and imaged using quantitative fluorescence image analysis (QFIA). It is respectfully submitted that Rao, et al., fail to teach or disclose, either expressly or inherently, the particular features and advantages of instantly claimed method.

First, the presently claimed method requires the use of at least two molecular markers that individually do not achieve sufficient specificity with regard to detecting cancer. It has been recognized that the use of particular single markers do not in many cases achieve sufficient specificity to distinguish between pathologically altered cells, e.g. cancerous cells or precancerous cells, from healthy cells, in part because the single marker may also be expressed in healthy tissues. And, the inventors have observed that, by simultaneously detecting at least two markers it is possible to compensate for the deficient specificity of the single marker so as to ensure a higher degree of specificity when detecting abnormal cells or tissues.

The markers proposed by Rao, et al., do not meet the claimed requirement in that they individually do not achieve sufficient specificity with regard to detecting cancer cells. More in particular, Rao, et al., propose to use G-actin and DNA content as biomarkers to evaluate cell samples for evidence of cancer or cancer risk. These markers, however, are clearly distinguishable from the claimed molecular markers. For example, G-actin, in contrast to the claims, is in fact capable of achieving sufficient specificity in detecting cancer cells when used individually, that is, by itself. Rao, et al., admittedly make this point on page 1031 wherein it is stated that "poorly differentiated cancer had significantly higher G-actin than well differentiated cancer" and that the difference was "statistically significant." Since G-actin alone is apparently sufficient to

detect differentiated cancer, it does not meet the requirement of the claimed molecular markers of the claimed method that the markers must not individually be capable of achieving sufficient specificity with regard to detecting cancer cells.

Similarly, the DNA content marker proposed by Rao, et al., do not meet the requirements of the claimed molecular markers because it is not unique to the detection of cancer. Rao, et al., visualize the DNA content of the cells by labeling the DNA with Texas-Red conjugated DNase I and then qualitatively assessing the structure and overall appearance of the DNA/nuclei using QFIA. However, the DNA content is also a molecular marker used in the analysis of non-cancer-related diseases, such as, for example, myocardial hypertrophy (see Matturri et al., "Characterization of myocardial hypertrophy by DNA content, PCNA expression and apoptotic index," International Journal of Cardiology, 82 (2002), pages 33-39, a copy of which is enclosed).

Rao, et al., teach only p53 as a marker that overlaps with the claimed method and consequently, Rao, et al., do not teach the requisite <u>at least two</u> molecular markers that individually do not achieve sufficient specificity with regard to detecting cancer. Accordingly, Rao, et al., do not teach each and every element of the claims.

Moreover, Rao, et al., do not teach or suggest the step of combining and accrediting the signal intensities of the detected markers in the cell or tissue sample. The claimed method involves the simultaneous detection of at least two markers in a cell or tissue sample wherein the identification of cancer is arrived at by quantitatively analyzing the combined signal intensities of the simultaneously detected markers. In contrast, Rao, et al., very clearly <u>do not</u> combine and accredit the signal intensities of its detected markers. Instead, as shown, for example, in Fig. 3, Rao, et al., <u>separately</u> analyze the detected signals of G-actin, p53 and DNA content. Fig. 3 shows a bar graph separately displaying the percentage of G-actin, p53 and DNA markers in benign as compared to PBDA and malignant breast tissues. Clearly, the signals are not combined and thus, the required features of the claims are not met.

Further still, Rao, et al., do not teach or suggest the step of automatically processing the signal intensities into image information and consolidating the information into a proposed diagnosis using a linked diagnostic expert system. While Rao, et al., appear to involve some automation with respect to the scanning of slide samples by QFIA, Rao, et al.,'s data analysis steps lack automation. As can be seen from page 1030-1031, Rao, et al., analyze the data beginning with a qualitative assessment of the morphology and staining patterns of the samples. Next, Rao, et al., compare and contrasts various quantitative fluorescence intensity data for the different markers used in order to make a determination as to the detection of cancer. No automation is involved in these determinations. This is significant because, unlike Rao, et al., the claimed method is concerned with objectively identifying cancer or cancer precursors in a sample on the basis of automatically processing the signal intensity information into a proposed diagnosis.

As detailed above, Rao, et al., fail to teach each and every element of the claimed method, either inherently or expressly. Since established case law requires that each and every element of a claim be described in a single prior art reference (*Verdegaal Bros. V. Union Oil Co. of California*, 814 F.2d at 631), Rao, et al., do not anticipate the presently claimed method. Accordingly, Applicants respectfully request reconsideration and withdrawal of the of the present rejection.

The Examiner has rejected claims 1, 3, 4, and 5 under 35 U.S.C. § 102(b) as being anticipated by McNamara, et al., (U.S. Patent No. 6,007,996) (Paper No. 2-29-2006, pages 3-4 and 6-7). Applicants respectfully traverse this rejection.

As noted above, established case law requires that for a claim to be anticipated, each and every element as set forth in the claim must be found, either expressly or inherently described, in a single prior art reference. *Id.* The following explanation will show that McNamara, et al., fail to teach each and every element of the presently claimed method and thus, the rejection under Section 102 in relation to McNamara, et al., should be reconsidered and withdrawn.

McNamara, et al., appears to relate to methods of pathological examination cells, for example, the detection of cancer cells, using multiple histological, immunohistochemical, and DNA ploidy stains that can be apparently examined simultaneously. However, no where does McNamara, et al., teach or disclose the step of combining and accrediting the signal intensities of the detected markers in the cell or tissue sample. The claimed method, as noted above, involves the simultaneous detection of at least two markers in a cell or tissue sample wherein the identification of cancer is arrived at by quantitatively analyzing the combined signal intensities of the simultaneously detected markers. While McNamara, et al., appears to relate to simultaneous detection of a plurality of molecular stains, it very clearly does not provide any teachings or suggestions to combine and accredit the signal intensities of its detected markers. At best, McNamara, et al., relates to the co-detection of a plurality of cellular stains where spectral images of separately-detected stains can be overlaid to produce a single, overlapping image (see e.g., Fig. 8). McNamara, et al., do not teach or suggest the claimed method because signal intensities of the detected markers are not combined in the way as required by the presently claimed method. And, an overlapping image does not constitute combining and accrediting signal intensities. This distinction is consistent with McNamara, et al.'s assertion that it is meant to "provide a pathologist with cumulative information regarding an examined biological sample and assist in the decision making" (see column 34, lines 35-37) rather than providing an objective determination of a proposed diagnosis, as in the claimed method.

Further still, McNamara, et al., do not teach or suggest the step of automatically processing the signal intensities into image information and consolidating the information into a proposed diagnosis using a linked diagnostic expert system. While McNamara, et al., appears to relate to the use of some automation

with respect to scanning slides, McNamara, et al., do not teach or suggest any step of automatically forming a proposed diagnosis based on processed signal intensities. This is a significant distinction because, unlike McNamara, et al., the claimed method is concerned with objectively identifying cancer or cancer precursors in a sample on the basis of automatically processing the signal intensity information into a proposed diagnosis. And, as noted above, McNamara, et al., essentially rely on the pathologist to, based on the available information, subjectively make the determination as to whether cancer is present.

Accordingly, as McNamara, et al., fail to teach each and every element of the presently claimed method, inherently or expressly, Applicants respectfully request reconsideration and withdrawal of the of the present rejection.

Rejection Under 35 U.S.C. § 103(a)

The Examiner has rejected claim 7 under 35 U.S.C. § 103(a) as unpatentable over either Rao, et al., or McNamara, et al., in view of U.S. Patent No. 5,109,429 (Bacus, et al.) (Paper No. 2-29-06, pages 4-5). Applicants respectfully traverse.

As amended, the claims are directed to automatable methods for identifying cancer cells and their precursors in a cell or tissue sample which comprises the steps of selecting at least two molecular markers that individually do not achieve sufficient specificity with regard to detecting cancer in said cells and contacting a cell or tissue sample with a signaling reagent that specifically binds to the at least two molecular markers. The claimed methods further comprise the steps of simultaneously detecting signals from the markers within a constituent region of the tissue section. Further, the signal intensities of the detected signaling reagent are combined and accredited, thereby identifying cancer cells and their precursors in the cell or tissue sample. The invention as defined by claim 7 is directed to a kit for implementing the method of claim 1 which comprises reagents for detecting molecular marker, auxiliary agents, reagents for detecting markers, controls, and protocols.

It is respectfully pointed out that "[t]o establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art." *See In re Royka*, 490 F.2d 981, 180 (CCPA 1974) and M.P.E.P. § 2143.03. Further, "[i]f an independent claim is nonobvious under 35 U.S.C. 103, then any claim depending therefrom is nonobvious." *See In re Fine*, 837 F.2d 1071 (Fed. Cir. 1988).

The Examiner separately combines Rao, et al., and McNamara, et al., with Bacus, et al., to allege in each case a *prima facie* case of obviousness. As pointed out above, however, neither Rao, et al., or McNamara, et al., teach or suggest each and every element of the presently claimed method. In particular, Rao, et al., fail to teach or disclose using at least two markers that individually do not achieve sufficient specificity with regard to detecting cancer cells. In addition, neither Rao, et al., nor McNamara, et al., teach or disclose combining and accrediting the detected signal intensities of the molecular markers such that

cancer or a cancer precursor may be detected in a cell or tissue sample. Further, neither reference teaches or discloses automatically processing the signal intensities into image information and consolidating the information into a proposed diagnosis using a linked diagnostic expert system.

Bacus, et al., do not cure the deficiencies of Rao, et al., or McNamara, et al. Bacus, et al., generally relates to a diagnostic testing apparatus for evaluating cells and tissues. The reference appears to especially relate to a diagnostic kit and a method for using the kit measuring and analyzing the quantity of cellular DNA and other cellular objects for the purpose of cancer diagnosis and prognosis. However, no where does Bacus, et al., teach or disclose the required features of the presently claimed method. Especially, Bacus, et al., do not teach or disclose using at least two markers that individually do not achieve sufficient specificity with regard to detecting cancer cells, combining and accrediting the detected signal intensities of the molecular markers, or automatically processing the signal intensities into image information and consolidating the information into a proposed diagnosis using a linked diagnostic expert system.

Rejected claim 7 depends from claim 1. As none of the cited prior art, either alone or in combination, render claim 1 *prima facie* obvious, it follows that the rejected dependent claim 7 is by extension nonobvious over the cited combinations of references. In other words, none of the deficiencies of Rao, et al., or McNamara, et al., are cured in any way by Bacus, et al., and thus none of the reference combinations meet the legal standard to render the claims obvious.

It is therefore submitted respectfully that Rao, et al., and McNamara., et al., either singly or in combination with Bacus, et al., fails to teach or suggest the method as presently claimed, and that the current invention is novel and nonobvious in view of the prior art references.

For the foregoing reasons in this section, Applicants respectfully request reconsideration and withdrawal of the present rejections.

Double Patenting

The Examiner has rejected claims 1, 2, and 4-7 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, 3, 4, 6, 9, and 10 of co-pending Application No. 10/022,618 (Paper No.20050610, pages 2-3).

It remains unknown what subject matter claimed and disclosed in the present application will be deemed allowable; hence any statement regarding this rejection made on Applicants' part would be premature. Therefore, Applicants respectfully traverse this rejection, and request that this rejection should be held in abeyance until subject matter is deemed allowable in this application.

CONCLUSION

For the foregoing reasons, Applicants submit that the claims are in condition for allowance and Applicants respectfully request reexamination of the present application, reconsideration and withdrawal of the present rejections and objections, and entry of the amendments. Should there be any further matter requiring consideration, Examiner Cross is invited to contact the undersigned counsel.

If there are any further fees due in connection with the filing of the present reply, please charge the fees to undersigned's Deposit Account No. 13-3372. If a fee is required for an extension of time not accounted for, such an extension is requested and the fee should also be charged to undersigned's deposit account.

Respectfully submitted,

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Characterization of myocardial hypertrophy by DNA content, PCNA expression and apoptotic index

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Abstract

Background: At present little is known about the biological basis of cellular alterations in myocardial hypertrophy. The present study aims to analyze proliferating cell nuclear antigen (PCNA) expression, DNA content and apoptosis, in several types of myocardial hypertrophy in order to define the biological characteristics of this process. *Methods:* The biological parameters were investigated in normal hearts ($n=4$) and in 21 cases of left ventricular myocardial hypertrophy related to pressure overload ($n=7$), post-infarction remodeling ($n=8$) and hypertrophic cardiomyopathy (HCM) ($n=8$). *Results:* The analyzed biomarkers were similar in hypertension and in remodeling, with a very high apoptotic index (mean values: 8.1 and 8.5%, respectively), a low PCNA positivity (mean values: 1.8 and 1.6%) and a prevalent diploid DNA content (DNA index: 1.2). Conversely, HCM showed a high mean PCNA index (21.2%) associated with a prevalence of hyperdiploid myocytes (DNA index: 1.8) and a low number of apoptotic cells (mean value: 1.7%). *Conclusions:* There are significant biological differences between hypertrophy in HCM and that related to arterial hypertension and post-infarction remodeling. Therefore, the combined evaluation of DNA content, PCNA and apoptotic indices could provide a powerful diagnostic tool in doubtful cases of myocardial primary or secondary hypertrophy and open new avenues in the clinical treatment of these entities.

Keywords: Myocardial hypertrophy; DNA; Proliferating cell nuclear antigen; Apoptosis

1. Introduction

Myocardial hypertrophy is characterized by an increase in myocyte size in the absence of cell division. It may be considered a compensatory or an adaptative process, that is, a response to pressure or volume overload (for various physiological and/or pathological reasons such as systemic hypertension, valvular malformations, etc.), loss of contractile mass (in myocardial infarction), or it may itself be a primary alteration, generally of unknown origin, as in hypertrophic cardiomyopathy [1–5].

At present little is known about the biological basis of cellular alterations in myocardial hypertrophy. Some studies, mainly experimental, have demonstrated that the increase in nuclear size observed in hypertrophic myocytes is related to an increase in DNA content [6–9]. The hyperdiploidy was observed also in our previous paper on hypertrophic cardiomyopathy (HCM) [10]. However, these ploidy increases are difficult to explain since, after birth and specially at termination of the physiological growth of the organism, myocardial tissue loses its ability to synthesize DNA [11–14].

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Proliferating cell nuclear antigen (PCNA) is a 36 kDa acidic, non-histone, nuclear protein required for DNA synthesis, and acts as the auxiliary protein of DNA-polymerase delta. Several studies carried out using PCNA antibodies have shown that PCNA accumulates in the cell nuclei during the S-phase and can be considered as a proliferation marker. Using a specific monoclonal method (PC10) has particular advantages over other techniques because of its relative simplicity and the rapidity of results [15].

The present study aims to analyze some biological parameters such as PCNA, DNA content and apoptosis, in various forms of myocardial hypertrophy (resulted from pressure overload, post-infarction remodeling and hypertrophic cardiomyopathy) in order to define the biological characteristics of this process and to determine whether differences exist in relation to the different pathologic conditions associated with it.

2. Materials and methods

The study was carried out in 21 cases of myocardial hypertrophy, obtained at necropsy from 13 males and eight females, aged from 38 to 88 years.

In eight cases hypertrophy was associated with left ventricular remodeling in post myocardial infarction, affecting the antero-lateral wall and/or the interventricular septum, in seven cases it was a consequence of arterial systemic hypertension and in eight it was related to HCM. Four of these cases were obstructive and four non-obstructive forms. An additional four hearts from three males and one female of 33, 51, 55 and 77 years respectively, who had not died from cardiovascular diseases, were used as controls.

For each case, five consecutive 5 μm sections were obtained from the left ventricle wall; fixed in buffered formaldehyde 10% and embedded in paraffin. Two of these were stained with hematoxylin/eosin and Azan for histologic examination, one was stained according to Feulgen for cytometric DNA analysis, and two were examined immunohistochemically for PCNA and apoptosis detection, respectively.

2.1. Cytometry

For evaluation of DNA content sections stained with Feulgen were examined. This method is based on the interaction between Schiff's reagent and the aldehyde groups of the deoxyribose molecules, previously unmasked by acid hydrolysis (5 N HCl at 22°C for 60 min) which removes the purinic bases.

Integrated optical density (IOD) was evaluated with a Zeiss CIRES image analyzer in 200 nuclei. The control reference value (diploid DNA content) was evaluated on the basis of 100 tissue lymphocytes. Nuclei that appeared to be overlapping or not clearly defined, were excluded from the assessment.

Ploidy is expressed as DNA Index=mean value ratio of DNA content of myocardial nuclei analyzed and that of reference lymphocytes.

DNA Index equal to 1 corresponds to diploidy, while DNA Index different from 1 corresponds to aneuploidy (value lower than 1 is hypodiploidy and higher than 1 hyperdiploidy).

2.2. PCNA evaluation

Sections were deparaffinized and brought to Tris–HCl-buffered saline solution (TBS; pH=7.6). After blocking endogenous peroxidase with 3% H_2O_2, the slides were incubated overnight with the primary antisera. Immunohistochemical staining was performed with the peroxidase–antiperoxidase method and avidin–biotin complex technique (ABC complex). Diaminobenzidine was used as chromogen substrate.

The sections were immunostained with PCNA monoclonal antibodies (DAKO) at a dilution of 1:200 and counterstained with light hematoxylin.

The PCNA index in every case was defined as the number of myocytes with strong unequivocal nuclear staining, corresponding to cells in S phase, divided by total number of cells counted, expressed as the percentage.

2.3. Apoptosis assay

The sections were deparaffinized and incubated with 20 μg/ml proteinase K. After the endogenous peroxidase treatment the deoxynucleotidyl transferase (TdT 0.3 U/ml), was used to incorporate digoxygenin-conjugated deoxyuridine (dUTP 0.01 mM/ml) to the ends of DNA fragments. The signal of TdT-mediated dUTP nick end labeling (TUNEL) was then detected by an anti-digoxigenin antibody conju-

gated with peroxidase. Apoptotic nuclei were identified by the presence of dark brown staining.

The apoptotic index was defined as the percentage of apoptotic nuclei on total number of myocardial cells evaluated in a minimum of 10 fields (×500). Of note, all the apoptotic cells counted were cardiomyocytes.

2.4. Statistical analysis

The differences between the mean values of ploidy, PCNA and apoptotic index in the different diseases studied and in normal hearts were calculated by variance analysis (F-test).

3. Results

Table 1 shows the densitometric and immunohistochemical results obtained for all cases studied.

The biological characteristics of myocardial hypertrophy secondary to hypertension and that associated with post-infarction remodeling were nearly the same. In both illnesses, the apoptotic index was very high (mean values 8.1 and 8.5%, respectively) with wide ranges of values, specially in the forms due to systemic hypertension (ranges: 0.7–16.6% and 1.5–11.9%) (Fig. 1). A different distribution of apoptotic nuclei was observed in both types. Apoptotic nuclei were widely distributed in cases of hypertrophy from volume overload but in cases of post-infarction remodeling they tended to be concentrated in fibrous areas rather than in the subendocardium.

On the other hand PCNA indices were low. For hypertensive hypertrophy the values ranged from 0 to 5.4% with a mean value of 1.8%. Similar PCNA results were obtained for post-infarction remodeling (range: 0–3.2%; mean value: 1.6%).

Regarding DNA content, analysis showed peaks mainly around diploidy with identical DNA index (1.2) and similar ploidy ranges (0.8–2.4 in hypertension and 0.9–1.8 in remodeling).

For HCM a contrasting opposite picture resulted. The apoptotic index was significantly lower than that observed in the other pathologic conditions (range: 0–4.5%; mean: 1.7%; $P<0.05$); conversely, the PCNA values were higher (range: 11.0–38.5%; mean: 21.2%; $P<0.05$) (Fig. 2) and DNA content almost tetraploid (DNA index=1.8).

In the control hearts the results were consistently lower than those observed in hypertrophy (mean values: PCNA=1.2%; apoptosis=0.9%; DNA=1.1). Of note, even if the expected number of mitotic cells is zero, a PCNA index positive value can exist in controls (see Discussion).

4. Discussion

The myocytes of the fetal heart and to some extent those of the neonatal heart have the ability to divide by mitosis [16–18]. Shortly after birth the myocardial tissue loses the ability to divide though it is still able to synthesize DNA.

In fact, the increase in heart size which accompanies the physiological growth of the organism occurs by hypertrophy of some of the pre-existing myocytes, which usually become hyperdiploid as it was stressed by other authors [1,11,12].

Hypertrophy may be more marked, affecting a large number of myocytes, as an adaptive or compensatory response in certain pathological processes resulting in an increase in cardiac mass [1–5]. However further clarification is required concerning the biological changes involved in hypertrophy, in particular the variations in the DNA content of

Table 1
PCNA, apoptosis and ploidy in hypertrophic and normal hearts

	PCNA index (%)		Apoptotic index (%)		DNA index	
	Range	Mean	Range	Mean	Range	Mean
Diseases						
HCM	11.0–38.5	21.2	0–4.5	1.7	1.0–2.6	1.8
Hypertension	0–5.4	1.8	0.7–16.6	8.1	0.8–2.4	1.2
Post-MI remodeling	0–3.2	1.6	1.5–11.9	8.5	0.9–1.8	1.2
Controls	0–3.0	1.2	0–2.5	0.9	0.9–1.8	1.1

HCM, hypertrophic cardiomyopathy; MI, myocardial infarction.

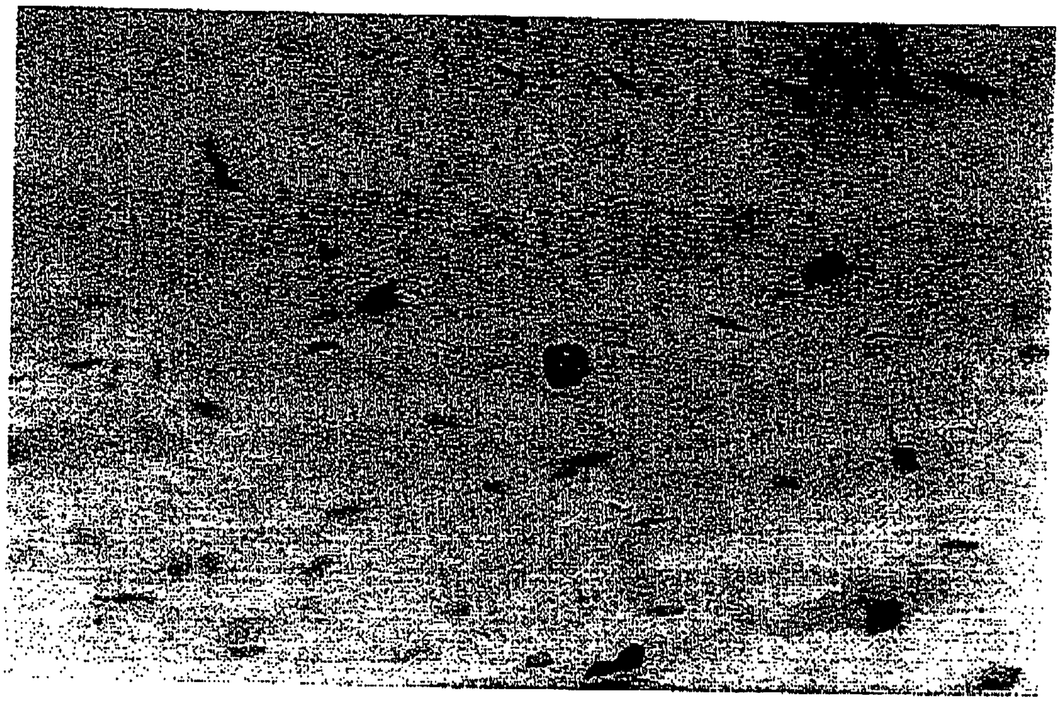

Fig. 1. High apoptotic index in secondary myocardial hypertrophy (×500).

myocytes, occurring outside the cell cycle, as demonstrated by the total absence of mitosis in the myocardium. Also hyperplasia could be the result of amitotic cell divisions of the nuclei, through longitudinal splitting of the hyperdiploid fibers, as shown by some authors [19–22] to be associated with a heart weight of over 500 or 250 g for the left ventricle.

Hypertrophy may be accompanied by qualitative and quantitative modifications of the expression of genes that codify the myocardial proteins [23–25]. Schaub et al. [26] have demonstrated, on cultured cardiomyocyte studies, that these modifications could be induced by factors such as anoxia, hormonal stimulation (thyroid hormones, catecholamines, angiotensin) and growth factors (Insulin-like Growth Factor, basic Fibroblast Growth Factor).

In the present study, by evaluating DNA content, PCNA and apoptosis, we aimed to characterize myocardial hypertrophy in different pathological conditions, namely arterial hypertension, post-infarction remodeling and hypertrophic cardiomyopathy.

Remodeling is the result of dynamic changes which affect the architecture of the left ventricle following myocardial infarction [27–30]. In this case, hypertrophy of viable myocytes quickly sets in, even within hours to few days of the acute event, and in segmental manner, mainly around the infarcted region [4,31,32]. On the other hand, hypertrophy that is secondary to arterial hypertension increases gradually, showing a homogeneous, concentric distribution [33].

However, in spite of the differences in the development process and also in the hypertrophic cell distribution pattern for post-infarction hypertrophy and that resulting from arterial hypertension, the biological picture which resulted in our study was similar in the two cases.

The most significant finding was the presence of a high apoptotic index in both forms of secondary hypertrophy, while the results related to PCNA index and DNA content were similar to those for normal heart myocytes.

Conversely, in the case of hypertrophic cardiomyopathy, the PCNA positivity was very high, and associated with a prevalence of hyperdiploidy and a low number of apoptotic cells.

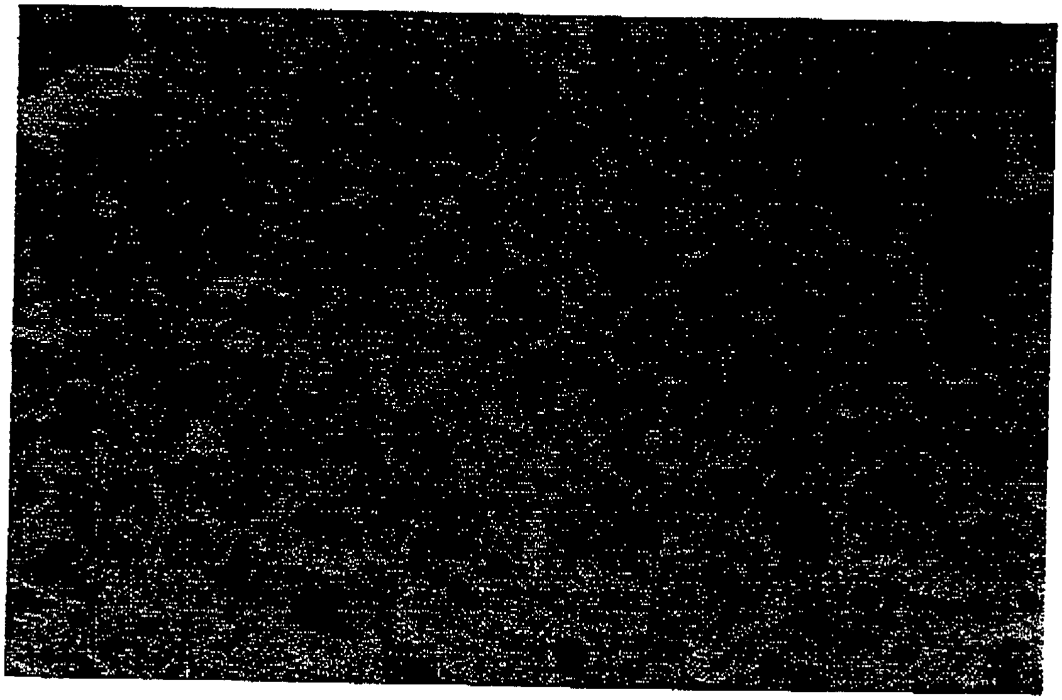

Fig. 2. High PCNA index in primary hypertrophic cardiomyopathy (×500).

The increased expression of PCNA in hypertrophic cardiomyopathy has been interpreted in one of our earlier studies [34] as a clear process of amitotic renewal of DNA, to counterbalance its marked instability. It may be stressed, that even if the expected number of mitotic cells is zero, a PCNA index positive value can exist in controls. In fact, the PCNA is a marker of the DNA content in interphase nuclei (particularly in the S phase) and it can be increased without the conclusion of the cell cycle in the mitosis. PCNA index is not equivalent of mitotic index.

A first consideration emerging from our present study concerns apoptosis. Although necrosis has been thought to be the principal form of cell death in the myocardium [35], we have observed that apoptosis is always present both in normal hearts and in pathological myocardial conditions, though the percentage of apoptotic myocytes varies, being lower in normal hearts and higher in the forms of hypertrophy resulting from pressure overload and infarction.

As apoptosis may play a compensatory role in tumor development [36–39], we believe that apoptosis could be an important regulator mechanism activated at the end of the hypertrophic process in order to eliminate the hyperdiploid myocytes. Conversely, Teiger et al. [40] have demonstrated in experimental studies that apoptosis appears in the early stages of myocardial hypertrophy. That is to say, its maximum value precedes the hypertrophic peak, which seems to be directly related to apoptotic cell fraction.

However, irrespective of the extent of apoptotic process and of the moment of onset, it can be considered that hypertrophy and apoptosis are two closely related processes. Moreover, some authors [41,42] have shown that when myocyte volume starts to increase the c-myc gene, an oncogene involved both in DNA synthesis and apoptosis, is expressed.

The most interesting feature of our study is the non-homogeneity of the results. It can be affirmed that, although the histologic picture for cardiac hypertrophy is very similar for the various diseases, with the exception of myocyte disarray in HCM, conflicting biological profiles nevertheless exist. In particular, there are substantial and significant differ-

ences between hypertrophy associated with hypertrophic cardiomyopathy and that related to arterial hypertension and to post-infarction remodeling.

If our data are confirmed by further studies involving a larger number of cases, the combined evaluation of DNA content and percentage of both PCNA and apoptotic positive cells could provide a valuable diagnostic tool, which would make it possible to distinguish between myocardial primary and secondary hypertrophy in doubtful cases. Of note, a thorough knowledge of the pathogenic mechanisms involved in these diseases may open new avenues in clinical treatments.

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